

The chloramphenicol resistance gene *cmlA* is disseminated on transferable plasmids that confer multiple-drug resistance in swine *Escherichia coli*

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Abstract

A recent study of β -hemolytic *Escherichia coli* isolated from diarrheic swine found that 53% were resistant to chloramphenicol, a drug that has been prohibited from use in food animals in the US since the mid-1980s. To identify the factors governing the persistence of chloramphenicol resistance in the absence of specific selection pressure, the location of the chloramphenicol resistance gene *cmlA* and its linkage to other resistance determinants were investigated. Southern blot analysis of plasmid DNA from 46 swine *E. coli* isolates indicated that *cmlA* was present on large plasmids greater than 100 kbp. Fifty-two percent of the isolates were able to transfer chloramphenicol resistance to an *E. coli* recipient at conjugation frequencies ranging from 10^{-3} to 10^{-8} per recipient. Antimicrobial susceptibility tests on transconjugant strains demonstrated that resistance to sulfamethoxazole, tetracycline, and kanamycin frequently transferred along with chloramphenicol resistance. The transconjugant strains possessed at least two distinct class 1 integrons that linked *cmlA* to both aminoglycoside resistance genes *aadA1* and *aadA2* and either to *sul1* or to *sul3* sulphonamide resistance genes. These results suggest that in the absence of specific chloramphenicol selection pressure, the *cmlA* gene is maintained by virtue of gene linkage to genes encoding resistance to antimicrobials that are currently approved for use in food animals.

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1. Introduction

Antimicrobials are valuable tools that animal producers use to quickly address clinical disease and to

maintain healthy and productive animals [1]. Nevertheless, broad use of antimicrobials in agriculture can select for resistant bacteria that may enter the food chain and potentially result in food-borne illness in humans that is less responsive to treatment with conventional antibiotics [2]. In addition, antimicrobial resistant pathogens pose a severe and costly animal health problem, as they prolong illness and decrease

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productivity through higher morbidity and mortality rates.

A central element of mitigation efforts to combat the increasing rates of antimicrobial resistance is the reduction of selection pressures through limited availability and decreased usage of antimicrobial drugs. In general, this strategy has enjoyed some success, such as in Europe, where the prevalence of resistance to macrolide and to glycopeptide antibiotics decreased in enterococci isolated from farm animals following the ban on related antimicrobial growth promoters [3,4]. However, there are reports of inconsistencies in the outcomes of this strategy. In recent Norwegian and Danish studies, vancomycin resistant enterococci persisted in broiler flocks after the ban of avoparcin for animal production, presumably because resistant strains survived extensive cleaning and disinfection of the broiler houses [5,6]. In Denmark, studies of cases in which vancomycin resistance was linked to erythromycin resistance reported that the prevalence of resistance to either drug did not decrease until the use of avoparcin and tylosin, agents chemically related to vancomycin and erythromycin, respectively, was limited [3]. And in a human clinical setting, a British study reported that sulfonamide resistance in *Escherichia coli* persisted in the United Kingdom despite a national prescribing restriction, most likely because of genetic linkage of multiple resistance determinants [7]. Knowledge of the molecular factors that govern the persistence of resistance in enteric bacteria in the absence of obvious selection pressure is however limited. Thus, there is a need to further determine the genetic location and potential linkage of antimicrobial resistance determinants among multi-drug resistant bacterial pathogens.

E. coli is a common etiologic agent that causes diarrhea in young pigs [8]. In the United States, broad spectrum antimicrobials such as streptomycin, tetracycline, and sulfamethoxazole are used for treatment of bacterial enteritis in livestock. Prior to the mid-1980s, chloramphenicol was commonly prescribed for treatment of colibacillosis, but concerns over its toxicity in humans led the US Food and Drug Administration to prohibit its use in food animals [9,10]. Nevertheless, a study on enterotoxigenic *E. coli* isolated in 1998 from diarrheic pigs reported that 53% of the isolates exhibited resistance to chloramphenicol [11]. Serotyping and ribotyping data indicated that the high rate of chloramphenicol resistance in swine *E. coli* isolates did not represent the clonal expansion of a single resistant strain but rather the dissemination of the chloramphenicol resistance gene *cmlA* among genetically diverse *E. coli* isolates. The present study investigates chloramphenicol resistance in pathogenic swine *E. coli* isolates with respect to the genetic location and mobility of associated resistance determinants.

2. Materials and methods

2.1. Bacterial strains and growth media

The present study focuses on a chloramphenicol-resistant subset ($N = 46$) of *E. coli* from a collection of β -hemolytic *E. coli* that were isolated from young diarrheic pigs and were described previously [11]. A nalidixic acid resistant strain of *E. coli* JM109, used as a recipient strain in conjugation experiments, was a gift from Dr. Shaohua Zhao, FDA Center for Veterinary Medicine, Laurel, MD. Tryptic soy broth (TSB) and tryptic soy agar (TSA) containing 5% sheep blood were purchased from Becton Dickinson, Sparks, MD. All cultures were grown at 37 °C for 24 h.

2.2. Analysis of plasmids

Analysis of plasmid DNA from transconjugant strains used a Qiagen Plasmid Mini Kit to isolate plasmid according to the manufacturer's instructions. Approximately 200 ng of plasmid DNA was digested with six units of *EcoRI* restriction endonuclease in 15 μ l of Multicore buffer (Promega Corporation, Madison, WI) for 1 h at 37 °C. Samples were applied to a 1% agarose gel, and subjected to electrophoresis for 3 h at 40 V. Contents of the gel were transferred by capillary action to a nylon membrane, fixed by UV irradiation, and analyzed by Southern blot analysis [12] using a digoxigenin labeled probe generated by polymerase chain reaction using *cmlA* specific primers [13]. A DIG Labeling and Detection kit (Roche Diagnostics Corporation, Indianapolis, IN) was used to label the probe and to detect hybridized targets according to the manufacturer's instructions.

2.3. Transfer of chloramphenicol resistance genes

Mobility of the chloramphenicol resistance genes was determined by the filter mating method described by Clewell and others [14]. Transconjugants were selected on TSA agar plates containing chloramphenicol (16 μ g ml⁻¹) and nalidixic acid (16 μ g ml⁻¹). Transconjugant colonies were sub-cultured on blood agar plates containing the relevant antibiotics and characterized with respect to antimicrobial susceptibility phenotypes.

2.4. Polymerase chain reaction

Polymerase chain reaction was used to detect the presence of genes encoding chloramphenicol resistance (*cmlA*), class 1 integrase (*intI1*), and sulphonamide resistance (*sul1*, *sul2*, and *sul3*). DNA templates were prepared by the boil lysis method, and PCR was performed using oligonucleotide primer sets specific for these genes as described previously [13,15,16]. To

determine the organizational structure of integrons containing *cmlA*, extra long PCR (GeneAmp XL PCR Kit, Applied Biosystems, Foster City, CA) was performed using the following primer sets: (1) *intII* forward and the *cmlA* reverse; (2) *cmlA* forward and *sulI* reverse; and (3) *cmlA* forward and *sul3* forward. Extra long PCR reactions (50 µl) contained: 1x XL Buffer II, 2.0 mM Mg(OAc)₂, 0.8 mM dNTPs, 1 pmol µl⁻¹ of each respective oligonucleotide primer, and three units *rTth* DNA polymerase. Reactions were subjected to 35 cycles of PCR with a denaturation temperature of 94 °C (1 min), an annealing temperature of 45 °C (0.5 min), and an extension temperature of 72 °C (10 min). Amplicons were submitted for DNA sequencing at the DNA Core Facility in the Department of Veterinary Pathobiology, Texas A&M University, College Station, TX. Sequence comparisons were made using the BLAST program available at the National Center for Biotechnology Information [17].

2.5. Determination of antimicrobial susceptibility

Antimicrobial susceptibility was determined by broth microdilution methods [18] using the Sensititre automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH). The following antimicrobials and resistance breakpoints (µg ml⁻¹) were used: amikacin (AMI), 64; ampicillin (AMP), 32; apramycin (APR), 32; amoxicillin/clavulanic acid (AUG), 32/16; cephalothin (CEP), 32; chloramphenicol (CHL), 32; cefoxitin (FOX), 32; kanamycin (KAN), 64; nalidixic acid (NAL), 32; sulfamethoxazole (SMX), 512; streptomycin (STR), 64; tetracycline (TET), 16; ceftiofur (TIO), 8. Breakpoints were determined using NCCLS interpretive standards [18] unless unavailable, in which case breakpoints in the National Antimicrobial Resistance Monitoring System 2001 Annual Report [19] were used. *E. coli* ATCC 25922 and 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms in all antimicrobial susceptibility tests.

3. Results and discussion

Studies in the United States and in Europe have reported persistent rates of chloramphenicol resistance years after withdrawal of the drug from therapeutic use in animals [11,20]. Since chloramphenicol is not approved for use in food animals in the United States, the high prevalence of the chloramphenicol resistance gene *cmlA* in swine *E. coli* is noteworthy [11]. Resistance to chloramphenicol may be mediated enzymatically through chloramphenicol acetyltransferase or non-enzymatically through the *cmlA* or *flo* genes, which both encode putative drug efflux pumps

[21,22]. The *flo* gene is similar in primary structure to *cmlA* and confers resistance to both florfenicol and chloramphenicol. It has previously been shown to be disseminated on large plasmids among genetically diverse strains of *E. coli*, and the use of florfenicol for treatment of respiratory infections in cattle may explain the selection of *flo* in bovine *E. coli* [23]. But neither chloramphenicol acetyltransferase nor *cmlA* confers resistance to florfenicol [24]. While it may be possible that florfenicol use may confer a low selection pressure for the *cmlA* gene, several studies in the literature suggest that persistence of resistance may be a result of the genetic linkage of resistance genes, where selection for one resistance marker selects for all markers [7,25]. The aim of the present study was to determine whether the persistence of chloramphenicol resistance in swine *E. coli* might be explained by genetic linkage of *cmlA* to other resistance genes.

3.1. Localization of *cmlA* and mobility of chloramphenicol resistance

We have examined a chloramphenicol-resistant subset (*N* = 46) of β-hemolytic *E. coli* isolates that were isolated between 1998 and 1999 and that possessed the chloramphenicol resistance gene *cmlA*. To determine the presence and size of plasmids carrying *cmlA* from swine *E. coli*, we used a modification of the S1 nuclease/pulsed field gel electrophoresis (PFGE) method described by Barton et al. [26]. Southern blot analysis using a *cmlA* specific probe detected a single *cmlA* target in 45 of 46 isolates, indicating that *cmlA* was present on large (>100 kbp) plasmids (data not shown). The absence of signal from strain 1581 would be consistent with a chromosomal location of *cmlA*, as the PFGE method used here to determine the size of plasmids does not detect chromosomal DNA.

Twenty-four of forty-six isolates (52%) were able to transfer chloramphenicol resistance to an *E. coli* JM109 recipient strain with conjugation frequencies ranging from 10⁻⁸ to 10⁻³ per recipient (Table 1). This suggests that conjugation of plasmids encoding *cmlA* is one mechanism for the wide dissemination of chloramphenicol resistance among swine *E. coli*. Interestingly, under the conditions employed in this study, all isolates belonging to the O149 and O139 serogroups transferred chloramphenicol resistance to the recipient *E. coli* strain, while none of those in the O147 serogroup did. Although there are few studies in the literature investigating the relationship between serogroup and plasmid conjugation, Danbara and co-workers reported a similar finding regarding serogroups of enterotoxigenic strains of *E. coli* isolated from human patients [27]. Strains from classical serogroups that were frequently associated with diarrhea were less likely to transfer enterotoxin plasmids to a recipient

Table 1

Conjugation frequency of chloramphenicol resistance and antimicrobial susceptibility of transconjugants

Strain	Conjugation frequency	MIC ($\mu\text{g ml}^{-1}$) of transconjugant ^a												
		AMI	AMP	APR	AUG	CEP	CHL	FOX	KAN	NAL	SMX	STR	TET	TIO
JM109	NA	16	4	≤2	4	8	16	8	≤16	>64	≤128	≤32	≤8	≤0.5
827	3.0×10^{-5}	8	4	≤2	4	8	>32	8	≤16	>64	>512	64	>16	≤0.5
828	7.1×10^{-5}	>32	≤4	>32	4	32	>32	>32	32	>64	>512	64	>16	2
829	1.0×10^{-8}	>32	4	>32	4	16	>32	>32	>64	>64	>512	64	≤8	1
830	2.8×10^{-7}	≤4	4	≤2	4	8	32	8	≤16	>64	>512	≤32	>16	≤0.5
839	3.3×10^{-8}	≤4	>32	≤2	4	16	>32	16	>64	>64	>512	≤32	≤8	≤0.5
843	1.0×10^{-7}	>32	4	>32	2	16	>32	>32	32	>64	>512	64	>16	2
844	3.2×10^{-6}	32	4	>32	4	16	>32	>32	32	>64	>512	64	>16	1
845	2.8×10^{-6}	8	8	32	4	16	>32	32	≤16	>64	>512	64	>16	2
850	1.1×10^{-5}	32	4	≤2	4	8	>32	32	>64	>64	>512	≤32	>16	≤0.5
851	7.4×10^{-4}	8	4	≤2	2	8	>32	16	>64	>64	>512	≤32	>16	≤0.5
860	8.0×10^{-5}	≤4	>32	≤2	8	8	>32	8	>64	>64	>512	64	≤8	≤0.5
862	2.7×10^{-6}	≤4	4	32	2	8	32	32	>64	>64	>512	≤32	16	≤0.5
1548	5.3×10^{-4}	≤4	>32	≤2	32	>32	>32	32	>64	>64	>512	64	>16	8
1550	2.4×10^{-4}	≤4	4	≤2	2	8	32	16	64	>64	>512	64	>16	≤0.5
1553	1.6×10^{-5}	≤4	4	≤2	4	8	>32	8	>64	>64	>512	≤32	>16	≤0.5
1557	4.1×10^{-3}	≤4	4	≤2	4	8	>32	8	≤16	>64	>512	≤32	≤8	≤0.5
1558	2.4×10^{-6}	32	4	>32	4	16	32	>32	>64	>64	>512	≤32	>16	≤0.5
1560	2.6×10^{-4}	8	4	16	4	16	>32	≤4	>64	>64	>512	≤32	>16	1
1561	1.5×10^{-4}	≤4	4	4	2	8	32	8	>64	>64	>512	≤32	>16	1
1562	1.6×10^{-5}	≤4	4	≤2	4	8	32	8	>64	>64	>512	≤32	>16	≤0.5
1566	8.4×10^{-6}	≤4	4	≤2	4	8	>32	8	>64	>64	>512	≤32	>16	≤0.5
1567	5.6×10^{-6}	≤4	4	8	2	8	>32	16	>64	>64	>512	≤32	>16	≤0.5
1580	1.5×10^{-5}	16	4	≤2	4	8	>32	32	>64	>64	>512	≤32	>16	4
1583	1.0×10^{-5}	≤4	4	≤2	4	8	>32	8	>64	>64	>512	≤32	>16	≤0.5

^a MICs that equaled or exceeded the resistance breakpoint are in bold.

than strains from serogroups that were rarely associated with diarrhea [28]. Strains within these serogroups may differ in many characteristics, including the presence of other mobilizing plasmids and fimbrial types; thus further work on a larger sample size would need to be conducted before evaluating the significance of this observation.

PCR analysis of total DNA from transconjugant strains indicated that the *cmlA* gene was transferred. None of the 24 donor strains possessed the chloramphenicol acetyltransferase gene *cat* or the florfenicol resistance gene *flo*, and therefore the presence of these genes in the transconjugant strains was not investigated. To confirm that resistance transfer occurred via conjugation of plasmids, plasmid DNA was isolated from the transconjugant strains, digested with the restriction enzyme *EcoRI*, and analyzed by Southern Blot using a *cmlA* specific probe. Comparison of restriction digest patterns between transconjugant strains indicated a genetically diverse set of plasmids (Fig. 1, upper panel). The Southern blot analysis was less complex, however, with the *cmlA* probe hybridizing to a single target of 5 kbp (18 of 24 strains), 7.5 (4 of 24 strains), and >12 kbp (1 of 24 strains) (Fig. 1, lower panel). In this analysis, *EcoRI* digestion of plasmid from transconjugant 1562 did not yield defined fragments; thus no *cmlA* target was detected by Southern blot. However, localiza-

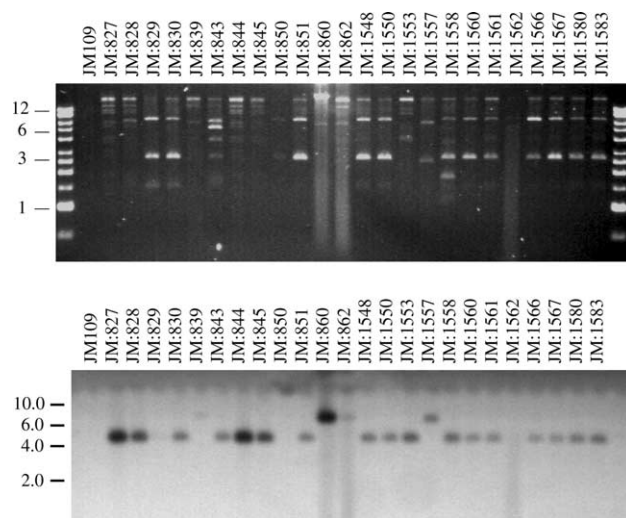


Fig. 1. *EcoRI* restriction digest of plasmid DNA isolated from transconjugant strains. Shown are the agarose gel stained with ethidium bromide (top panel) and the Southern blot analysis using a DIG-labeled *cmlA* probe. Sizes (kilobase-pairs) of molecular standards are indicated on the left.

tion of *cmlA* to plasmid DNA from transconjugant 1562 was confirmed in a separate Southern blot analysis following digestion with the restriction enzyme *NotI* (data not shown).

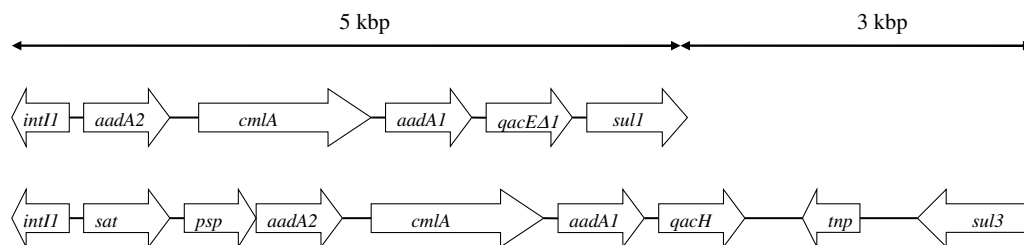


Fig. 2. Organization of class 1 integrons of swine *E. coli*. Gene cassettes are indicated by block arrows. The upper integron is from isolate 862 and the lower integron is from isolate 1562. Sequence data for each integron is available in the GenBank database with Accession Nos. **AY816215** (isolate 862) and **AY816216** (isolate 1562).

3.2. Antimicrobial susceptibility of transconjugant strains

Transconjugant strains derived from chloramphenicol resistant swine *E. coli* donors were tested for susceptibility to other antimicrobial agents. Co-transfer of resistance to at least one other antimicrobial was observed in all transconjugants (Table 1). The predominant co-transferring resistances were to the following agents: sulfamethoxazole (24 of 24 transconjugants), tetracycline (21 of 24 transconjugants), and kanamycin (17 of 24 transconjugants). This is consistent with results from the initial study of chloramphenicol resistance in swine *E. coli*, which reported a statistically high level of significance for co-resistance to these same agents [11]. Since sulphonamides, aminoglycosides, and tetracycline are commonly used in swine in the United States, it supports the hypothesis that a genetic linkage between *cmlA* and resistance genes to these agents ensures persistence of the chloramphenicol resistance phenotype, even in the absence of a direct chloramphenicol selection pressure.

3.3. PCR analysis of DNA from transconjugant strains

Integrons are genetic structures consisting of two conserved segments that flank a central region where cassettes of resistance genes may accumulate. In general, class 1 integrons consist of antibiotic resistance gene cassettes flanked by the integrase gene *intI1* and the sulfonamide resistance gene *sul1* [29,30]. The co-transfer of sulphonamide resistance in all 24 transconjugants suggested that *cmlA* may be part of an integron. PCR analysis of total genomic DNA confirmed the presence of the class 1 integrase gene *intI1* in all transconjugant strains (data not shown). However, only four transconjugant strains (829, 839, 860, and 862) possessed *sul1*. Extension of the analysis to screen for related sulphonamide resistance genes *sul2* and *sul3*, found that the remaining 20 transconjugant strains possessed *sul3*. The *sul3* gene, which encodes a putative dihydropteroate synthase with 40% sequence identity to *sul1*, was first reported in 2003 [31]. Subsequent studies have documented its prevalence among *E. coli* from both animal and human sources in

Europe, but it has not previously been reported in strains isolated in the United States [32,33].

Extra-long PCR using the *intI1*-forward and the *cmlA*-reverse primers detected specific amplicons in all 24 transconjugant strains (data not shown). Two distinct amplicons were detected: a 2.5 kbp amplicon in the four strains that possessed *sul1*, and a 4.0 kbp amplicon in the remaining strains. Analysis using the *cmlA*-forward primer and the *sul1*-reverse primer on transconjugant strains 829, 839, 860, and 862, yielded a 3.0 kbp amplicon. Similar analysis on the remaining 20 strains using *cmlA*-forward and *sul3*-reverse failed to produce an amplicon. Use of the *cmlA*-forward and the *sul3*-forward primers, however, gave a 5 kbp amplicon, indicating that the *cmlA* gene and *sul3* gene are in opposite orientations.

As representatives of each distinct integron, the amplicons from transconjugant strains 862 and 1562 were sequenced. The organization of the two distinct integrons is shown in Fig. 2. The structure of the integron from strain 862 is similar to that of *P. aeruginosa* plasmid R1033 transposon Tn1696 (GenBank Accession No. **U12338**), with *cmlA* in close proximity to the aminoglycoside resistance genes *aadA2* and *aadA1* and to *sul1*. The structure of the integron from strain 1562 is unique, as there are presently no sequences in the GenBank database that possess all of the following genes in order: the streptothricin resistance gene *sat*, the putative phosphoserine phosphatase gene *psp*, *aadA2*, *cmlA*, *aadA1*, the quaternary ammonium compound resistance gene *qacH*, and *sul3*. The 5'-end of the 1562 integron containing the *sat*, *psp*, and *aadA2*, is similar to one reported from a strain of *E. coli* O159 isolated in Japan [34]. The *cmlA* and *aadA1* domain is similar that described for the *P. aeruginosa* plasmid R0133. The original characterization of *sul3* on plasmid pVP440 reported that a truncated *cmlA* gene was localized downstream and in the same orientation of *sul3* [31]. No other sequences in the database report the linkage of *sul3* to other resistance gene cassettes or as part of an integron. Thus the data presented here not only describes a unique integron structure, but also demonstrates a direct genetic link between *cmlA* and genes encoding aminoglycoside and sulphonamide resistance.

It is worth noting that while the transconjugant strains possessed aminoglycoside resistance genes, not all were resistant to streptomycin. The concentration of streptomycin in the antimicrobial susceptibility panels used in this study ranged from 32 to 256 $\mu\text{g ml}^{-1}$. All nine streptomycin resistant transconjugant strains had an MIC for streptomycin (resistance breakpoint = 64 $\mu\text{g ml}^{-1}$) equal to 64 $\mu\text{g ml}^{-1}$, while the MIC in the susceptible strains could only be measured as $\leq 32 \mu\text{g ml}^{-1}$. The streptomycin-susceptible phenotype in fifteen strains, despite possessing the *aadA2* gene, may simply be a matter of what breakpoint is used, since there is no NCCLS approved interpretive criteria. Nevertheless, this data demonstrates a direct genetic link between *cmlA* and genes encoding aminoglycoside resistance enzymes.

4. Conclusion

This study illustrates the complexity of resistance dynamics, which must be taken into account when developing effective intervention strategies. Our results suggest that a mechanism for co-selection and maintenance of chloramphenicol resistance in pathogenic swine *E. coli* exists in the absence of direct selection pressure from phenicol use. Since co-resistance to sulfamethoxazole, tetracycline and kanamycin was observed among the majority of chloramphenicol resistant transconjugants, the use of any of these antimicrobials can result in the selection of bacteria resistant not only to that specific agent, but by genetic linkage of resistance genes, to other unrelated antimicrobial agents, in this case chloramphenicol. Therefore, the simultaneous reduction in the selection pressures of all co-selecting agents may be required to mitigate the dissemination of antimicrobial resistance in the animal production environment.

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